PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 5/10, 5/06, 15/87 A61K 35/54, 35/12, 48/00 A61F 2/02, 2/10	A1	(11) International Publication Number: WO 91/16419 (43) International Publication Date: 31 October 1991 (31.10.91)

(21) International Application Number: PCT/US91/02629

(22) International Filing Date: 17 April 1991 (17.04.91)

(30) Priority data:
513,290 17 April 1990 (17.04.90) US

(71) Applicant: EROX CORPORATION [US/US]; 535 Middlefield Road, Suite 240, Menlo Park, CA 94025 (US).

(72) Inventors: BERLINER, David, L. ; 380 Selby Lane, Atherton, CA 94027 (US). STENSAAS, Larry, J. ; 2460 Lynwood Drive, Salt Lake City, UT 84109 (US).

(74) Agent: CIOTTI, Thomas, E.; Irell & Manella, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: METHODS OF TISSUE IMPLANTATION

(57) Abstract

This invention discloses a method of treating a mammal in need of tissue implantation by providing a developing amniote egg that has a chorioallantoic membrane (CAM); implanting mammalian cells or tissue on the CAM so as to produce a conditioned mammalian tissue; removing the conditioned tissue from said CAM by a means of excision; and reimplanting the conditioned tissue in the mammal. The invention also discloses related compositions and methods of treatment.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MG**	Madagascar
AU	Australia	**FI**	Finland	**ML**	Mali
BB	Barbados	**FR**	France	**MN**	Mongolia
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Faso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GN**	Guinea	**NL**	Netherlands
BJ	Benin	**GR**	Greece	**NO**	Norway
BR	Brazil	**HU**	Hungary	**PL**	Poland
CA	Canada	**IT**	Italy	**RO**	Romania
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		

METHODS OF TISSUE IMPLANTATION

Technical Field

This invention relates generally to methods of implanting and reimplanting mammalian tissue in mammals. More specifically, the invention relates to: procedures which increase implantation efficiency; methods of treatment which involve implantation; and tissue compositions useful for implantation.

Description of Related Art

The chorioallantoic membrane (CAM) is a heavily vascularized, extraembryonic, fetal membrane of birds. It provides essential gas exchange for the developing avian embryo. Due to its easy access and proximal separation from the embryo it has been studied and utilized by both developmental and tumor biologists.

Beginning in the early part of this century, the CAM was commonly used to study the growth of small tumor transplants (Karnofsky et al, Ann NY Acad Sci (1952) 55:313. More recently, it has been used in studies of membrane transport, gas exchange, and angiogenic activity. The CAM has also been used in developmental studies as a substrate for the growth of many types of embryonic avian tissues and organ primordia (Rawles, Ann NY Acad Sci (1952) 55:302). Ebert cultured organ explants from post-hatch chickens on CAM tissue to study the effects of the explants on the corresponding tissues in the developing egg (Proc Nat Acad (1954) 40:337; J Nat Cancer Inst (1960)

2:73). Hurst et al has reported that some normal human tissue grown on the CAM showed mitotic activity (Austr J Exp Biol (1939) 17:215), however contrary reports have indicated that adult endothelial tissue does not survive more than three days when implanted on the CAM (Ausprunk & Folkman, Virchows Arch B Cell Path (1976) 21:31).

Implantation as a method of treatment has found many applications in medicine. However, the widespread use of implantation has always been limited by the efficiency of survival of the implanted tissue.

For example hair transplantation is used to treat alopecia (hair-loss). Scalp baldness may be treated by surgical transplantation of active hair follicles to the scalp.

Recent reports have suggested that the symptoms of Parkinsonism can be ameliorated by autogenic implantation of adrenal medullary tissue to the striatum of the brain, but the transplanted tissue survives for only a relatively short time (Ding, Y.J. et al, Chin Med J, (1988) 101:631; Hansen, J.T. et al, Exp Neurol (1988) 102:65; Stromberg, I. et al, Exp Brain Res(1985) 60:335). Most recently, Brundin et al (Exp Brain Res(1988) 70:192) has reported that human fetal dopamine neurons can be similarly used in rats; and Lindvall et al (Arch Neurol (1989) 46:615) has reported some success in humans.

Implantation is an important step in the early development of mammalian embryos. Techniques of in vitro fertilization are being more widely applied, employing both immediate and cryogenically delayed implantation (Camus, M. et al, Fert and Steril (1989) 51:460), but again implantation efficiency has proven to be a limitation (Liu, H.C. et al, Fert and Steril

(1988) 49:649). Wiemer et al (Fert and Steril (1989) 52:503) has reported that implantation efficiency increases if human zygotes are co-cultured on a monolayer of fetal bovine uterine fibroblasts.

Finally, gene therapy has been proposed as a method of treatment for various metabolic deficiencies as well as illness which require the chronic administration of ameliorative proteins (Green, H. et al, Proc Nat Acad Sci (1979) 76:5665; Palmer et al, Proc Nat Acad Sci (1987) 84:1055), but again the successful reimplantation of genetically engineered tissues presents a limitation of the technique.

Summary of the Invention

One embodiment of the invention is a method of treating a mammal in need of tissue implantation which comprises: providing a developing amniote egg that has a chorioallantoic membrane (CAM); implanting mammalian cells or tissue on or in the CAM so as to produce a conditioned mammalian tissue comprising a population of cells; removing the conditioned tissue from the CAM by a means for excision; and, implanting the conditioned tissue in the mammal.

In another embodiment developing chicken eggs of the genus Gallus are used; in yet another embodiment developing ostrich eggs of the genus Struthio are used.

In another embodiment, the invention is used to treat both genetic and metabolic deficiencies of gene expression. In still another embodiment, the invention is used to treat alopecia. In other embodiments, the invention is used to treat Parkinsonism.

In yet another embodiment, the invention is used to facilitate the implantation of mammalian preimplantation embryos into the uterine epithelium of recipient mothers. In another embodiment, the invention provides a method of in vitro fertilization of mammalian oocytes. In a final embodiment, the invention is a composition of tissues useful for mammalian implantations which composition comprises mammalian tissue and growth factors derived from an avian CAM.

Detailed Description of the Invention

Definitions

An "allogeneic" implant comprises cells or tissue which have a different genetic constitution than the host tissue; this because they are obtained from a subject other than the recipient. An "autogenic" implant comprises cells or tissue originating within the body of the recipient (with the same genetic constitution). Finally, a "homotypic" implant comprises cells or tissue of the same genetic constitution and the same type and function.

"Amplified" tissue is substantially increased in mass as a result of mitotic growth.

An "amniote egg" has a shell and retains its own water supply when external to the parent. It contains a yolk and abundant albumin. The embryo develops within the egg, but external to the parent. Examples include avian and reptile eggs.

"Conditioned tissue" has been grown on the CAM and, as a result, is amplified and substantially vascularized.

A "developing" egg is a fertilized amniote egg which has begun ontogenesis in response to the appropriate conditions of incubation.

A "fertilization unit" is comprised of a mammalian embryo, usually at the blastocyst stage of development, and mammalian uterine epithelial tissue into which the embryo has been implanted. Prior to the implantation of the embryo, the uterine epithelial tissue has been implanted beneath the choroid epithelium of the CAM of a developing amniote egg such as a chicken or ostrich egg. The fertilization unit is subsequently reimplanted in vivo into the uterine epithelium of the mammalian mother.

"Implantation" is the moving of tissue from one site to another and embedding the moved tissue such that it remains viable. Tissue can be implanted at a new site in the original subject or in a heterologous recipient. The implanted tissue can be placed within tissue of similar function or of different function.

A subject which is "in need of" a medical or surgical procedure is one who has an injury, disease, deficiency or medical condition that would likely be ameliorated by the procedure; or alternatively, a subject whose normal physiologic process (e.g. embryo implantation during pregnancy) would be facilitated by the procedure or function.

A "neurological impairment" is an injury, disease or degeneration of nerve cells or nervous tissue resulting in a loss of nerve function.

A DNA "structural sequence" encodes a polypeptide.

"Transplantation" is a form of implantation in which the tissue being moved is united with the

same tissue-type in another place. If the transplant is from one part of the body to another part it is an "autotransplantation" which is equivalent to a homotypic implant.

"Vascularized" tissue has developed new blood vessels which are patent to the circulation of the host.

Modes of Carrying Out the Invention

Several injuries, diseases and deficiencies are treatable by tissue implantation and transplantation. In addition, some normal physiologic processes such as mammalian pregnancy involve tissue implantation. The effectiveness of these treatments and the success of these processes is directly related to the efficiency of implantation of the newly introduced tissue as measured by the sustained growth and differentiated function of the implanted tissue. Disclosed herein are methods of conditioning tissue to increase the efficiency of implantation; treating a mammal in need of implantation with the conditioned tissue; and compositions of tissues useful for such treatment.

Briefly, the methods of conditioning tissue, and its subsequent use in treatment, involve providing an amniote egg with an embryo that has developed a chorioallantoic membrane (CAM), and implanting mammalian cells or tissue, which are intended to be subsequently implanted in the recipient mammal, onto or into the CAM. The tissue grows and is amplified on or in the CAM as the embryo continues to develop, because patency is established between the circulatory system of the egg and the vasculature of the implant; and because the embryonic environment is rich in

nutrient and growth factors. In addition, the implant tissue becomes hyper-vascularized in response to embryo angiogenic factors. The conditioned mammalian tissue is subsequently excised, along with adherent portions of the CAM, and implanted in a location in the mammalian host which is best suited to the particular treatment regime. The efficiency of implantation is greatly improved, relative to direct tissue implantation, due to the hypervascularization of the implanted tissue, and the tendency of the adherent embryonic vessels to rapidly form functional connections with capillaries of the host tissue. Furthermore, growth factors, provided by the embryonic tissue, may also accompany the implant and promote its integration into the transplantation site.

While this procedure can utilize any amniote egg, in a preferred embodiment, an egg of the class Reptilia or Aves is used. In a more preferred embodiment, eggs of the genus Gallus (chicken) are used. Fertile chicken eggs which have been examined and certified free of pathogens are readily available from commercial sources. Methods of artificial incubation are known to those skilled in the art. In another preferred embodiment, eggs of the genus Struthio (ostrich) are used. While eggs certified pathogen-free are not presently available commercially, ostriches and eggs are commercially available (Big Sky Ostrich Ranch, Plentywood, Montana) and the eggs can be monitored to meet the same standards that are presently used for chick eggs. These methods are generally known to those skilled in the art. Methods of artificial incubation of ostrich eggs are also known (Jarvis, M.J.F., Ostrich (1985) 56:42).

The technology of preparing the chicken egg and the CAM to receive a tissue implant is well known to those skilled in the art (See e.g. Zwilling, E., Transplant Bull, (1959) 6:115). The same technology can be generally applied to the amniote eggs of other species with appropriate changes for different gestation periods. This technology has been modified to extend the survival of the implant and enable its subsequent reimplantation in a mammalian host. This modified technique is described below.

A fertile chicken egg, which is pathogen free, is obtained and incubated at 37 degrees Celsius in a humidifier chamber (incubator) for 6 to 9 days prior to implantation of mammalian tissue. The age of the chick embryo determines the amount of mammalian tissue to be implanted. A larger amount of implant tissue requires a more vascularized CAM surface and an increased pumping capacity of the chick heart (an older chick embryo), which in turn provides a more adequate flow of blood.

The air space at one end of the egg is identified by holding the egg above a small, low intensity light source (candling) and a small (one millimeter) hole is made to allow air to enter the air space. The embryo and the extra-embryonic membranes are then identified, and an "x" is placed with a lead pencil over the mid-portion of the air space to mark its location. A 1 centimeter square window is outlined in the shell centered around the "x" by inscribing lines by means of a small triangular file. The grooves so created are progressively deepened to the egg membrane below. The surface of the egg is cleaned by wiping the area around the "x" with a cotton swab containing 80% ethanol solution for a few

seconds and the surface is allowed to dry. The window is then removed by inserting the tip of a pair of sterile watchmaker's forceps under one corner of the square and lifting to expose the underlying egg membrane.

The egg membrane is carefully penetrated with the tip of the sterile watchmaker's forceps to permit air to enter so that the CAM will detach and "drop". This membrane is then incised with a sterile scalpel and carefully removed by tearing it away from the perimeter of the window to expose the CAM. The surface of the CAM is inspected at low magnification under a dissecting microscope in order to identify the principal vessels that are then traced to a point of convergence where they form the allantoic vascular supply to the embryo. A provisional site is identified near the convergence of the vessels on the CAM surface for the implantation of mammalian tissue, and the choroid epithelium gently abraded with the tip of a sharp glass needle to provide one or more implantation sites. The window is temporarily closed by placing a small piece of Scotch brand tape over the aperture.

An implant of living tissue is secured under sterile conditions from a deeply anesthetized mammal. Small pieces of live tissue are secured (not exceeding 1 millimeter x 3 millimeters x 1 millimeter) by rapid dissection. The tissue is either immediately placed onto the previously identified CAM implantation site or quickly transferred into a cold (4 degrees celsius) sterile culture solution situated in a Petri dish.

The cold solution favors survival of cells in the tissue by reducing its metabolic requirements and by permitting diffusion of leukotrienes and

proteins induced by injury which would otherwise create a local inflammatory reaction on the CAM.

The tissue then undergoes final trimming to remove surrounding connective tissue and reduction to the desired size. This step is critical to insure the rapid fusion of the choroid vessels with microvasculature elements exposed at the surface of the implant. This is discussed further infra.

The egg is then opened by removal of the tape cover, and the previously identified implantation site is positioned under the egg center of the window by gently rotating the egg. The small pieces of tissue to be implanted are removed from the Petri dish by means of sterile forceps placed gently under each piece, and lifted out of the culture medium. Excess culture medium is rapidly blotted by resting the forceps on sterile absorbent paper, and then delivered to the CAM with a minimal residual quantity of fluid remaining on the surface.

The tissue is gently placed on the surface of the CAM implantation site and carefully moved over the previously abraded surface to secure good contact without inducing additional bleeding from the underlying small capillaries of the choroid surface. In the event that an undue quantity of culture media remains at the CAM implant surface, it is removed by "wicking away" the liquid using the tip of a sterile cellulosic sponge (e.g., tissue paper).

A variety of procedures may be employed to improve the "receptivity" of the CAM which would favor rapid fusion of the choroid vessels with microvascular elements exposed at the surface of the implant. In additional to superficial laceration of the CAM

surface in order to decrease the time required for vascular fusion.

Critical to survival of the implant is the rapid fusion of vessels which will provide the implant with blood from the circulatory system of the developing embryo. This fusion is executed within a matter of minutes by micro-vascular elements within the egg which identify the exposed micro-vascular elements of the implant. Little is known of the dynamics involved in this recognition process, but it is assumed to depend, in part, on the superficial location of the extra-embryonic coelomic vessels immediately below or with the choroid epithelial. Furthermore, the abraded choroid epithelial cells may move away from points of contact with the implanted tissue and to thereby expose underlying vascular elements of the choroid.

Vascular fusion is sometimes also favored by covering the implant with a small (5 mm x 5 mm) sterile, transparent plastic "chip" (Gel-Bond, FMC Corp.) whose weight and hydrophyllic surface promote contact with the CAM while preventing drying of the implant.

Following implantation the window of the egg is closed with tape and the edges are sealed by means of paraffin wax applied by means of a hot scalpel blade. The eggs are then incubated at 37 degrees celsius. Implants may be inspected by placing the egg under the stereoscopic microscope, removing the tape window, and rotating it so the implant is visible. Photographs may also be taken at this and prior stages using the stereoscopic microscope. At high magnification it is possible to determine the viability of the implant by visualizing blood flow in small surface

vessels which develop at the implant - CAM interface within one or two days after implantation. Such vessels continue to increase in size with time, and certain types of tissue (i.e., adrenal medullary tissues) can be distinguished by virtue of its high degree of vascularization.

Re-implantation of the conditioned implants into a mammalian host is effected by anesthetizing the recipient, and, using standard surgical procedures known to those skilled in the art, exposing a portion of the target organ provided with a non-traumatized micro-vascular bed. In the case of the brain, stereotaxic procedures, known to those skilled in the art, are employed by which the tissue can be delivered into the proper location with minimal trauma.

The implants are readied for reimplantation by first cooling them (while still in the egg) in a refrigerator for a few minutes. The shell window is then opened and approximately 1 to 2 cubic centimeters of cold culture media is used to irrigate the implant surface. After 20 to 30 seconds the culture media is aspirated to expose the implant, and the underlying choroid membrane is carefully stripped away.

In a preferred embodiment, the implant is then removed from the remaining allantoic-mesenchyme surface by means of carefully controlled "aspiration" using a large gauge hypodermic needle whose tip has been modified to provide a sharpened, round, non-oblique aperture. Suction for such aspiration is provided by a tightly fitting "plunger" consisting of a second filled needle which can be withdrawn to create a vacuum. The aspirated tissues in the lumen of the larger (outer) needle shaft can then be safely conveyed to the reimplantation site in the mammalian

host where it can be deposited either on the surface or within the prescribed tissue. In an alternate embodiment, the implant is remove by a circumscribed incision followed by a trimming of surrounding tissue.

Of course, it is essential to carry out all of the above reimplantation procedures using sterile technique. It should be also mentioned, however, that a peculiar resistance to infection seems to exist for tissue implanted into the egg. This has so far failed to be reported by other investigators. Thus, it is possible to achieve survival of the implant in the egg without the maintenance of absolute sterility. On the other hand, similar resistance to infectious agents is unlikely to occur for tissue reimplanted into a mammalian host. It is therefore essential to use maximal sterile precautions when engaging in the reimplantation procedures.

The following examples are intended to illustrate but not limit the invention.

Example 1

In the Treatment of Alopecia

Alopecia (hair-loss) can presently be treated by removing skin plugs from a hair-covered skin area of a balding subject and implanting them in the areas of baldness. This approach is limited because a balding subject has an ever-shrinking reservoir of appropriate hair-covered skin.

Dermal papillae contain cells capable of inducing hair growth, and unknown growth factors, arising from cells in dermal papillae, must be continuously provided to sustain hair growth. Baldness arises from a loss of hair due to a prolonged period of inactivity of the stem cells in the papillae

of particular follicles which give rise to the cells required for hair growth and maintenance. The approach disclosed herein involves elaborating stem cells derived from dermal papillae of active hair follicles on or in the CAM of a chicken egg. Growth of these stem cells on the CAM creates a reservoir of highly active autogenic stem cells and provide a substantial mass of tissue, that is transplantable to areas of baldness.

This autotransplanted tissue is implanted under inactive follicles and subsequently exerts a stimulatory effect on the endogenous stem cells of the dermal papillae, and on related keratinocytes, resulting in hair growth.

Example 2

In the Treatment of Human Infertility

The techniques commonly referred to as in vitro fertilization include the collection of oocytes from a donor (which may also be the recipient) the fertilization in vitro of at least one oocyte, and its subsequent reimplantation on or in the uterine epithelium of the recipient mother. One of the most difficult hurdles in this procedure is the reimplantation step. Only one in six (or more) attempts results in a fetus that goes to term.

To improve the efficiency of reimplantation, the fertilized egg is first allowed to implant into uterine epithelial implant tissue (taken from the recipient mother) which is in the process of growing on the CAM. After implantation and establishment of the embryo in this epithelial implant, the entire fertilization unit is then reimplanted into the mother.

Briefly, a small explant of uterine epithelium is obtained either before or at the time of egg recovery and is grown "stroma-down" on the CAM. While growing on the CAM the uterine epithelium can be prepared to receive the fertilized blastocyst by hormonal conditioning. After fertilization in vitro, the mammalian embryo is transferred to the surface of the uterine tissue explant and allowed to implant. The implantation can be certified by visual inspection. When growth and implantation is assured, the entire fertilization unit, including its highly vascular stroma would be microsurgically reimplanted back into the uterine wall of the mother.

The efficiency of embryo implantation is increased due to the ability to condition the epithelium without exposing the recipient to systemic hormonal treatment, an additional advantage results from the fact that the success of implantation can be ascertained prior to insertion of the embryo into the recipient, thereby avoiding unnecessary patient trauma.

Example 3

In the Treatment of Human Infertility

In an alternate embodiment the zygote (fertilized oocyte) is cultured in a small plastic enclosure within the extraembryonic coelom of the CAM rather than first culturing uterine epithelial tissue on the CAM and subsequently implanting the blastocyst into this cultured tissue. The advantage of this approach is that it keeps the entire fertilization unit small (<3mm) which improves the ultimate in vivo reimplantation efficiency.

Example 4

In the Treatment of Human Infertility

In an alternate embodiment the oocyte is fertilized directly on the CAM rather than in a culture dish or test tube. A 2mm hole covered with a 3mm gold screen is situated in a sterilized, transparent, hydrophyllic plastic chip (Gel Bond, FMC Corp.) about 3-7mm in diameter. The choroid epithelium is then removed from a 2mm x 2mm site by gentle abrasion with a recently fine-polished glass rod without producing bleeding. The plastic chip containing the gold-covered hole is then placed over the abrasion site on the CAM and the hole allowed to fill with the coelomic fluid (free of blood). Then the oocyte together with its adherent cumulus (coronal) cells is placed in the hole. While the oocyte matures it is treated with hyaluronidase which greatly reduces the number of coronal cells and exposes the oocyte surface. After the oocyte has matured, spermatozoa are then added and co-incubated with the oocyte so as to permit fertilization.

The advantage of this approach is that the embryonic environment of the CAM is more conducive to successful fertilization and subsequent survival of the fertilized zygote than is a test tube environment. This increases the overall efficiency of the in vitro fertilization procedure.

Example 5

In the Treatment of Parkinsonism

In this embodiment, the method of culturing mammalian tissue on the avian CAM, described supra, is used to condition tissue derived from autogenous adrenal medulla that is to be used for brain

transplants to treat human subjects suffering from Parkinsonism. Here too, the lack of survival of the implant at its point of insertion has emerged as the principal limitation to success of the procedure (See e.g. Ding, Y.J. et al, Chin Med J (1988) 101:631; Hansen, J.T. et al, Exp Neurol (1988) 102:65). The surgical techniques of adrenal medullary explantation and striatum implantation is known to those skilled in the art (Id.).

Using the standard protocol of immediately transplanting adrenal tissue, the critical initial step involving fusion of capillaries at the surface of the transplant with those of this brain simply does not occur rapidly enough and extensively enough to provide for tissue survival and growth. By greatly amplifying the number of surface capillaries during the period of CAM implantation, and by the conditioning and proliferation of the parenchymal tissue itself, transplant survival is greatly enhanced upon subsequent reimplantation into the brain graft sites.

Example 6

In the Treatment of Parkinsonism

In this embodiment, the tissue to be implanted in the striatum of a subject afflicted with Parkinsonism is derived from human fetal tissue. More specifically, dopamine producing neurons from the ventral mesencephalic region of abortus brain tissue is implanted onto the CAM of an avian embryo. 5 to 10 days later, the conditioned fetal tissue is excised from the CAM and implanted into the striatum of a human subject suffering from Parkinsonism. Stereotaxic techniques are used to assure proper placement. These techniques are well known to those

skilled in the art. The patients are treated with a cyclosporine, azathioprine, and steroid regime to minimize the risk of graft rejection.

Example 7

In Somatic Cell Gene Therapy

Using gene transfer technology known to those skilled in the art, a gene is inserted into autologous somatic cells of a patient suffering from a disease characterized by a deficiency of a naturally occurring gene product (e.g. diabetes and insulin) or a disease characterized by its ameliorative response to the introduction of a gene product (e.g. heart disease and tissue plasminogen activator).

The choice of somatic cell type within which the treatment gene is inserted is governed by the mode of delivery desired: e.g. transduced epithelial keratinocytes are used to provide a "skin patch" from which the gene product is secreted; transduced endothelial cells are introduced into vascular grafts to provide direct secretion of a gene product into the circulatory system; and transduced parenchymal cells (such as hepatocytes) provide organ-specific introduction of a gene product.

In each case, genetically engineered (e.g. transformed or transduced) cells are first introduced onto the CAM of a developing avian embryo and cultured for 5-10 days, in the case of a chick egg, or 10-20 days in the case of an ostrich egg. During this culturing, the introduced cell population expands and forms a tissue which then becomes vascularized. This conditioned tissue is subsequently excised and homotypically reimplanted in the desired location

within the patient using the techniques described supra.

Autogenic cells which have been genetically engineered may also be used in combination with the other methods of treatment described supra. For instance, adrenal medulla cells can be genetically altered to up-regulate the production of dopamine, prior to their implantation on the CAM and subsequent reimplantation in the brain of Parkinson patients.

CLAIMS

What is claimed is:

1. A method of preparing mammalian tissue for implantation in a mammalian subject which comprises:

providing a developing amniote egg that has a chorioallantoic membrane (CAM);

implanting mammalian cells or tissue on or in the CAM so as to produce a conditioned mammalian tissue comprising a population of cells; and

removing the conditioned tissue from the CAM by a means for excision.

2. The method of claim 1 in which the mammal in need of implantation is human.

3. The method of claim 1 in which the amniote egg is from the class Aves.

4. The method of claim 3 in which the egg is from the genus Gallus.

5. The method of claim 3 in which the egg is from the genus Struthio.

6. The method of claim 1 in which the excision means is aspiration.

7. The method of claim 1 in which the excision means is incision.

8. The method of claim 1 in which the tissue is autogenic.

9. The method of claim 8 in which the tissue is homotypic.

10. The method of claim 8 in which the genome of a subpopulation of cells of the tissue is genetically altered.

11. The method of claim 9 in which the genome of a subpopulation of cells of the tissue is genetically altered.

12. The method of claim 11 wherein the tissue implantation is for the purpose of providing synthesis of a polypeptide in the subpopulation of cells and the genetic alteration comprises: the insertion of DNA comprising a structural sequence encoding the polypeptide; and the structural sequence being functionally linked to DNA control sequences.

13. The method of claim 12 in which the polypeptide synthesis is needed to ameliorate a genetic deficiency.

14. The method of claim 8 in which the tissue implantation is for the purpose of treating alopecia, the cells implanted on the CAM comprise stem cells derived from dermal papillae of active hair follicles of the subject, and the conditioned tissue is implanted into the dermis of the subject at a site where hair growth is desired.

15. The method of claim 8 in which the tissue implantation is for the purpose of treating Parkinsonism and the conditioned tissue is implanted into the striatum of the brain of the subject.

16. The method of claim 15 in which the tissue is derived from an adrenal medulla of said subject.

17. The method of claim 1 in which the implanted tissue is allogeneic.

18. The method of claim 17 in which the tissue implantation is for the purpose of treating Parkinsonism, and the tissue is derived from a human fetal tissue and is implanted into the striatum of the brain of the subject.

19. The method of claim 8 in which the tissue implantation is for the purpose of treating a neurologic impairment.

20. The method of claim 19 in which the tissue is derived from neurologic tissue of the subject and is implanted at the site of the impairment.

21. A method of implanting a mammalian embryo into epithelial tissue of the uterine wall of a mammalian mother comprising:

providing a developing amniote egg that has a chorioallantoic membrane (CAM);

implanting an explant of uterine epithelial tissue from the mother on or in the CAM for a given time to produce a conditioned epithelium;

placing the mammalian embryo on or in the conditioned epithelium;

incubating the embryo and the conditioned epithelium until the embryo implants in the conditioned epithelium, thereby forming a fertilization unit;

excising the fertilization unit from the CAM; and

implanting the unit back into the in vivo uterine epithelium of the mother.

22. The method of claim 21 used in conjunction with an in vitro fertilization of the ovum.

23. The method of claim 21 in which the fertilization unit is incubated for greater than 1 day but less than 20 days prior to implantation of the unit into the uterine epithelium of the mother.

24. A method of implanting a mammalian embryo into epithelial tissue of the uterine wall of a mammalian mother comprising:

providing a developing amniote egg that has a chorioallantoic membrane (CAM);

implanting a mammalian blastocyst onto or into the CAM;

incubating the blastocyst for a given time;

excising the blastocyst from the CAM; and

implanting the blastocyst into the in vivo uterine epithelial tissue of the mother.

25. The method of claim 24 in which the blastocyst is incubated in or on the CAM for greater than 1 day but less than 20 days prior to implantation into the uterine epithelium of the mother.

26. A method of fertilizing a mammalian oocyte comprising:

providing a developing amniote egg that has a chorioallantoic membrane (CAM);

abrading a portion of the choriod epithelium of the CAM;

preparing a transparent, hydrophillic plastic chip containing a hole covered with a gold screen;

placing the chip on the abraded epithelium;

placing at least one mammalian oocyte into the hole;

introducing spermatozoa of the same species into the hole; and

co-incubating the oocyte and spermatozoa for a given period of time.

27. The method of claim 26 in which the oocyte is treated with hyaluronidase prior to the introduction of spermatozoa.

28. A composition of tissue useful for mammalian implantation comprising a mammalian tissue intended to be implanted into a mammalian host, and growth factors derived from avian CAM tissue; the mammalian tissue having been first implanted in or on the CAM of a developing amniote egg for a given time such that the mammalian tissue has become substantially amplified, vascularized and conditioned as a result of the implantation.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/02629

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

U.S. Cl.: 435/1, 240.1, 240.241; 424/561,570,572,574,582; 600/34; 623/11

IPC(5): C12N 5/10, 5/06, 15/87; A61K 35/54, 35/12, 48/00; A61F 2/02, 2/10

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/1,204.1, 240.2, 240.21, 240.241; 424/561,570,572, 574/581,582,93; 600/34; 623/11,15; 514/44

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

CAS, BIOSIS, MEDLINE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	NUZZOLO ET AL, "CELL AND TISSUE CULTURE TECHNIQUES" PUBLISHED 1983 BY WARREN H. GREEN, INC. (MO), SEE PAGES 111-113.	1,3,4,28 2,5-27
P,Y	US,A, 4,980,286 (Morgan et al) issued 25 December 1990, see the entire document.	10-14
Y	FERTILITY AND STERILITY, Volume 52, No.3, issued September 1989, Wiemer et al, "Coculture of human zygotes on fetal bovine uterine fibroblasts: embryonic morphology and implantation", pages 503-508, see entire document.	21-28
Y	AUST.J.EXP.BIOL., Volume 17, issued 1939, Hurst et al, "A note on the survival and growth of human and rabbit tissues (normal and heoplastic) on the chorioallantois of the chick and duck embryo", pages 215-224, see the entire doucment.	1-28

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

05 JULY 1991

Date of Mailing of this International Search Report

13 AUG 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

J.M. STONE

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EXP. NEUROL., Vol. 102, issued 1988, Hansen et al," Adrenal Medullary Autografts into the Basal Ganglia of Cebus Monkeys: Graft Viability and Fine Structure", pages 65075, see the entire document.	15,16,18-20